Copper(II)-*bis*(thiosemicarbazonato) complexes as antibacterial agents: insights into their mode of action and potential as therapeutics

Karrera Y. Djoko[1#], Maira M. Goytia[2], Paul S. Donnelly[3], Mark A. Schembri[4], William M. Shafer[5], and Alastair G. McEwan[6#]

[#]Corresponding authors

[1]School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, QLD 4072, Australia. Email: k.djoko@uq.edu.au

[2]Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322. Email: mgoytia@emory.edu

[3]School of Chemistry and Bio21 Institute of Molecular Science and Biotechnology, The University of Melbourne, Parkville, VIC 3010, Australia. Email: pauld@unimelb.edu.au

[4]School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, QLD 4072, Australia. Email: m.schembri@uq.edu.au

[5]Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322. Laboratories of Bacterial Pathogenesis, Veterans Affairs Medical Center, Decatur, GA 30033. Emory Antibiotic Resistance Center, Emory University School of Medicine, Atlanta, GA 30322. Email: wshafer@emory.edu

[6]School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, QLD 4072, Australia. Email: mcewan@uq.edu.au

ABSTRACT

There is increasing interest in the use of lipophilic copper (Cu)-containing complexes to combat bacterial infections. In this work, we showed that Cu complexes with *bis*(thiosemicarbazone) ligands [Cu(btsc)] exert antibacterial activity against a range of medically significant pathogens. Previous work using *Neisseria gonorrhoeae* showed that Cu(btsc) complexes may act as inhibitors of respiratory dehydrogenases of the electron transport chain. We now show that these complexes are also toxic against a range of bacterial pathogens some of which lack a respiratory chain. Respiration in *Escherichia coli* was slightly affected by Cu(btsc) complexes but our results indicate that, in this model bacterium, the complexes act primarily as agents that deliver toxic Cu ions efficiently to the cytoplasm. Although the chemistry of Cu(btsc) complexes may dictate their mechanism of action, their efficacy depends highly on bacterial physiology which is linked to the ability of the bacterium to tolerate Cu and additionally, the susceptibility of the respiratory chain to direct inhibition by Cu(btsc) complexes. The physiology of *N. gonorrhoeae*, including multidrug-resistant strains, makes it highly susceptible to damage by Cu ions and Cu(btsc) complexes, highlighting the potential of Cu(btsc) complexes as a potential treatment against this significant bacterial pathogen.

Keywords: copper, ionophores, antimicrobial, Cu(btsc), *Neisseria gonorrhoeae*

INTRODUCTION

Copper (Cu) is an essential trace micronutrient in bacteria but it is bacteriotoxic in excess. Nutrient Cu inserts into specific high-affinity sites in proteins, or it forms complexes with low molecular weight thiols such as glutathione. As a consequence, the concentration of "free" Cu ions in cells has been estimated to be vanishingly low (sub-femtomolar) (1). Any excess Cu beyond this normal binding capacity may be mislocated to nonspecific and low-affinity metal ion binding sites, thereby inactivating crucial enzymes and interfering with normal metabolism (2). As the mid-potential of the Cu(II)/Cu(I) redox couple is biologically accessible, these weakly bound Cu ions can also cause further toxicity by catalyzing gratuitous electron transfer and promoting redox stress (3). The widespread damage caused by excess Cu ions is termed Cu poisoning. Almost all bacterial species, including those that have no apparent use for Cu, possess homeostatic systems that protect them against an excess of this ion (4). These systems consist typically of a Cu ion efflux pump that operates under the control of a Cu-specific transcriptional regulator.

As a result of its chemistry, Cu is considered a promiscuous or broad-spectrum antimicrobial but there is concern regarding its universal toxicity. In the pre-antibiotic era, simple ionic Cu salts were used to treat a variety of infections (5), but these salts are now associated with significant toxicity (6). The difficulty in delivering bactericidal Cu at doses that are nontoxic to human tissues may explain the failure to translate Cu-based therapeutics into the modern medical setting. The emergence of bacteria with resistance to classical antibiotics, combined with the paucity of new compounds in the pipeline, has now renewed interest in the development of Cu as an antimicrobial (7-9). This research is motivated further by recent

findings that physiological Cu may be harnessed as a direct antimicrobial in innate immune cells (10, 11).

Ionic Cu salts have limited ability to cross membranes as their translocation relies on specific transport proteins, and so they have little potential to be developed as anti-infective drugs. Cu ions generally show restricted penetration into target tissues and bacteria, and thus exceptionally high concentrations are needed to achieve a bactericidal effect *in vitro* and *in vivo*. To assist in the delivery of Cu ions across lipid membranes, several lipophilic ligands or proligands have been developed (9, 12). These molecules are termed 'Cu ionophores' for their ability to act as carriers of Cu ions.

Of interest in this work are *bis*(thiosemicarbazone) ligands that bind Cu(II) as small, uncharged, lipophilic, and stable complexes (Cu(btsc), Figure 1). We and others have shown that Cu(btsc) complexes exert antimicrobial activity against several human pathogens, including Gram-negative bacteria such as *Neisseria gonorrhoeae* (13) and *Mycobacterium tuberculosis* (14), as well as the Gram-positive bacterium *Staphylococcus aureus* (15). However, the mechanism of antibacterial action of Cu(btsc) complexes has not been examined fully. Although it is assumed that they operate as Cu ionophores (14, 15), we have shown that Cu(btsc) complexes can also act as direct respiratory inhibitors in *N. gonorrhoeae* (13). Here we describe experiments using *Escherichia coli* that are aimed at determining whether Cu(btsc) complexes act primarily by inhibition of the respiratory chain or by delivery of bioavailable Cu ions. We also assess the susceptibility of a range of bacterial pathogens to these complexes, including multidrug-resistant strains of *N. gonorrhoeae*.

MATERIALS AND METHODS

Cu stocks. Stocks of aqueous Cu salts, supplied as $CuCl_2$ or $Cu(NO_3)_2$, were prepared in deionised water and their concentrations were standardised using bathocuproine disulfonate as described elsewhere (16). The two salts were used interchangeably in experiments. Stocks of Cu-pyrithione, Cu-neocuproine, and Cu-disulfiram complexes were prepared in DMSO by mixing standardised $CuCl_2$ with 2.5 molar equivalents of the respective free ligands. Stocks of Cu(atsm) and Cu(gtsm) were prepared in neat DMSO and standardised using their solution absorbance in DMSO (Cu(atsm): λ_{max} 457 nm, ε 7200 M^{-1} cm^{-1}; Cu(gtsm): λ_{max} 472 nm, ε 8700 M^{-1} cm^{-1}). DMSO was used as a vehicle control in all experiments.

Strains and growth conditions. *N. gonorrhoeae* strains (Table 3) were grown on GC agar (Oxoid) supplemented with 1 v/v % IsoVitaleX (Becton Dickinson). Liquid cultures were prepared in GC broth supplemented with IsoVitaleX and 0.042 w/v % sodium bicarbonate. *E. coli* strains MG1655 (laboratory strain K-12) and EC958 (fluoroquinolone-resistant strain ST131), and *Salmonella enterica* sv. Typhimurium SL1344 were propagated on LB agar or broth. *Haemophilus influenzae* RdKw20 was propagated on BHI medium (Oxoid) containing 10 μg/mL NAD^+ and 10 μg/mL hemin. *Streptococcus pneumoniae* D39 was propagated on THY base (Oxoid) containing 10 U/mL catalase. *S. aureus* strain ATCC 22913 (methicillin-sensitive), and SR2852 (methicillin-resistant) strains were grown on Tryptic Soy medium (Becton Dickinson). *Lactobacillus acidophilus* NCFM was grown on *L*-MRS agar (Oxoid) under anaerobic growth conditions. All bacteria were grown for 12 – 16 h at 37 ^oC. *N. gonorrhoeae*, *S. pneumoniae*, and *S. aureus* were grown in the presence of 5 v/v % atmospheric CO_2. Anaerobic growth was performed in an anaerobic jar containing an AnaeroGen sachet (Oxoid).

Determination of MICs. MICs were determined by agar dilution method. Briefly, bacterial lawns from an overnight agar culture were resuspended in the appropriate broth to 10^7 – 10^8 CFU/mL and 5 μL of each dilution was spotted onto new solid media containing various concentrations of the desired Cu source. The amount of DMSO was maintained at 0.5 v/v %. The MIC value was defined to be the minimum concentration at which no growth was visible after 24 h.

Assays of gonococcal killing kinetics. *N. gonorrhoeae* lawns from an overnight agar culture were resuspended in broth to an OD_{600} of 0.1 (ca. 10^7 CFU/mL). Cu complexes were added to the desired final concentration and the mixtures were incubated at 37 ^oC with gentle shaking. Survival was monitored for up to 7 h by conventional colony counting.

Measurement of intracellular Cu levels in *E. coli*. *E. coli* was resuspended in broth to an OD_{600} of 0.1 (ca. 10^8 CFU/mL) and used to seed fresh solid media containing the desired Cu source. After an overnight growth, bacteria were harvested, washed once in PBS containing 10 v/v % DMSO, once in PBS containing 1 mM EDTA, and finally once in PBS without any additive. The final pellet was dissolved in conc. nitric acid. Amounts of Cu and other transition metal ions were determined by Inductively-Coupled Plasma Optical Emission Spectrometry (ICP OES). Results were standardised to total biomass as represented by total protein content. The amount of protein was assayed using the Quanti BCA Kit (Sigma).

To evaluate the antibacterial activity of the Cu complexes under the same conditions, the initial inoculum was diluted serially up to 10^{-7} and 5 μL of each dilution was plated out on duplicate solid media containing the various Cu sources. Visible colonies were enumerated after 24 h to determine the efficiency of plating.

Assays of β-galactosidase activity in *E. coli*. The promoter region of *copA* from *E. coli* MG1655 (150 bp upstream from the translation start codon ATG) was amplified using the primer pairs P*copA*-F (5'-CATCAGGGATTCAGATAAATGTCTAATCCTGA-3') and P*copA*-R (5'-CTGATGAAGCTTAAAACACTCCTTTAAGACAG-3'). The PCR product was cloned between the *Bam*HI and *Hind*III sites of the low-copy vector pQF50 containing a promoterless *lacZ* gene (17). The resulting pQF50::P*copA* plasmid was transformed into *E. coli* DH5α. Colonies that were blue on agar plates containing ampicillin (100 μg/mL), X-gal (40 μg/mL), and $CuCl_2$ (500 – 1000 μM) were selected and grown to the mid-exponential phase. Cultures were challenged with Cu complexes for 0 – 120 min without any shaking. β-galactosidase activities were measured using *ortho*-nitrophenyl-β-galactoside following procedures for standard Miller assay. Raw values of β-galactosidase activity were normalised to OD_{600}. Results were expressed as fold-induction over the uninduced control at the same time point.

Assays of respiration in *E. coli*. *E. coli* was cultured to the mid-exponential phase, harvested, resuspended in LB to 10% of the original volume, and kept on ice. Respiration was measured at 35 ^{o}C in 2 mL of broth containing 50 μM of Cu salt, Cu(gtsm), or Cu(atsm). Consumption of O_2 was initiated by adding 10 μL of the bacterial suspension. Respiration was monitored for 15 min using an S1/Mini Clark-type oxygen electrode (Hansatech Instruments) in conjunction with an Oxytherm control unit.

E. coli membrane vesicles were isolated as described previously for *N. gonorrhoeae* (13). Rates of NADH oxidation were measured spectrophotometrically at 340 nm following procedures described previously for submitochondrial particles from rat liver (18).

Assays of using cervical epithelial cells. Immortalised and adherent ME-180 cervical epithelial cells (ATCC® HTB33™) were cultured routinely according to manufacturer's

157 instructions in McCoy's 5a Modified Medium (ATCC 30-2007) containing fetal bovine serum
158 (10 v/v %, ATCC 30-2020), and penicillin and streptomycin (100 IU/mL and 100 μg/mL,
159 respectively, Corning-Cellgro 30-002-CI), Cells were seeded at 1 x 10^6 cells/mL and allowed to
160 adhere overnight. The resulting monolayers were transitioned into fresh medium containing
161 Cu(gtsm), Cu(atsm), or Cu salt. The concentration of DMSO in all samples was 0.1 v/v %. After
162 24 h, the supernatant was collected and checked for cells that may have detached during
163 treatment. The remaining adherent cells were allowed to recover for 1 – 2 h and subsequently
164 brought into suspension using trypsin/EDTA. Trypan Blue (0.2 w/v %) was added and cells were
165 enumerated in a hemocytometer.
166
167 **RESULTS**
168
169 **Antibacterial activity of Cu(btsc) complexes towards select bacterial pathogens.**
170 Two prototypes of the Cu(btsc) family, Cu(gtsm) and Cu(atsm) (Figure 1), exerted dose-
171 dependent antibacterial activity against *N. gonorrhoeae* strain 1291 (13). Both complexes were
172 effective at concentrations where the unmetallated H_2btsc ligands and the uncomplexed or 'free'
173 aqueous Cu^{2+} ions were ineffective (Table 1) (13). The MICs were 0.1 μM (0.03 μg/mL) for
174 Cu(gtsm) and 1 μM (0.3 μg/mL) for Cu(atsm) (Table 1). We also evaluated the anti-gonococcal
175 activities of additional Cu ionophores, including Cu-disulfiram, Cu-neocuproine, and
176 Cu-pyrithione. The MIC value for Cu-pyrithione (0.13 μM, ca. 0.04 μg/mL) was comparable to
177 that for Cu(gtsm) (Table 1). For this study, we examined Cu(gtsm) in greater depth as the
178 availability of the structural analogue Cu(atsm) with a lower efficacy allowed us to probe the
179 mode of action in more detail.

The mode of action of Cu(gtsm) and Cu(atsm) against *N. gonorrhoeae* was bactericidal. Complete killing of *N. gonorrhoeae* (~10^7 CFU/mL) by 1 μM of Cu(gtsm) was achieved within 1.5 h, while an equal dose of Cu(atsm) required at least 5 h to elicit an equivalent effect (Figure 2). By contrast, ionic Cu salts displayed no bactericidal activity within this time period (Figure 2). The relative MICs and killing kinetics established that Cu(gtsm) and Cu(atsm) were more toxic than Cu salts, and that Cu(gtsm) was more toxic than Cu(atsm).

Cu(gtsm) and Cu(atsm) were also effective against several additional and unrelated bacterial pathogens, including *S. aureus* (methicillin-sensitive and resistant strains), *S. pneumoniae*, and *H. influenzae*, but at MICs that were appreciably higher (Table 2). *E. coli* (fluoroquinolone-sensitive and resistant strains) and *S.* Typhimurium were the most resistant. In the case of *E. coli*, bacterial growth was observed even at Cu(gtsm) concentrations as high as 25 μM (7.5 μg/mL), although there was a 1000-fold reduction in the plating efficiency when compared with the untreated control (Figure S1 in Supplemental Material). In comparison, exposure to 25 μM of Cu(atsm) had no effect (Figure S1). Due to limited solubility of Cu(btsc) complexes, higher concentrations were not tested.

Since *N. gonorrhoeae* and *E. coli* represented the most susceptible and most resistant test organisms, respectively, they were examined further; the former because it may be a promising target for Cu- and Cu(btsc)-based therapeutics and the latter because its resistance properties and its amenability to molecular analysis might help in determining the mode of Cu(btsc) action.

Inhibition of respiration in *E. coli* by Cu(btsc) complexes and antibacterial activity under anaerobic growth conditions.

202 Recently, we showed that Cu(gtsm) and, to a lesser extent, Cu(atsm) suppressed aerobic
203 respiration in *N. gonorrhoeae* (13) and mitochondria (18). Within the electron transport chain,
204 NADH dehydrogenases (Nuo or Complex I (H^+-translocating) and Nqr (Na^+-translocating)) were
205 identified as the primary targets of inhibition. Inhibition occurred at or near the site of
206 ubiquinone reduction and it was independent of the release of 'free' Cu ions (18). Instead, an
207 intact Cu(gtsm) or Cu(atsm) molecule was determined to be the inhibitory species. Subsequently,
208 we proposed that the action of Cu(btsc) complexes as respiratory inhibitors may be a major
209 mechanism of their antibacterial activity (13). However, our present work has now shown that
210 these complexes were also effective against bacteria that do not respire such as *S. pneumoniae*
211 (Table 2).

212 Cu(gtsm) (50 μM) also suppressed respiration in *E. coli*, as indicated by a decrease in the
213 total amount of O_2 consumed after 15 min, while equal concentrations of Cu salts or Cu(atsm)
214 had no effect (Figure 3A). *E. coli* possesses two respiratory NADH dehydrogenases, Nuo and
215 Ndh-2 (single-subunit flavoenzyme, does not translocate H^+) (19), which may be targets of
216 Cu(gtsm) inhibition. However, Cu(gtsm) only weakly suppressed the rates of NADH oxidation
217 in isolated membrane vesicles containing both Nuo and Ndh-2 (Figure 3B). The I_{50} value was
218 extrapolated to be >130 μM (Figure S2), well beyond the solubility limit for Cu(gtsm) indicating
219 that the NADH dehydrogenases in *E. coli* were not major targets of respiratory inhibition by
220 Cu(gtsm). This finding was not altogether surprising, as Nuo and Ndh-2 in *E. coli* were also less
221 sensitive to inhibition by the classical Nuo or Complex I antagonist, rotenone. This difference
222 has been ascribed to subtle structural differences at or near the sites of ubiquinone reduction (20).

223 More importantly, Cu(gtsm) was also inhibitory to *E. coli* under anaerobic growth
224 conditions, as evidenced by a curve of plating efficiency that was essentially indistinguishable to

225 that obtained under aerobic growth conditions (Figure S1). Therefore, unlike the situation in *N.*
226 *gonorrhoeae* (13), inhibition of aerobic respiration did not appear to contribute significantly to
227 the antibacterial activity of Cu(gtsm) against *E. coli*.
228
229 **Boosting of intracellular Cu levels by Cu(btsc) complexes.**
230 The MICs for both Cu(gtsm) and Cu(atsm) were consistently lower than those for ionic
231 Cu salts by >2 orders of magnitude (Table 2). Unlike charged Cu ions, neutral Cu(btsc)
232 complexes are presumed to be readily membrane-permeable. Thus, treatment with Cu(btsc)
233 complexes would be expected to drive a greater accumulation in bacterial Cu contents when
234 compared with equal doses of Cu salts. However, previous analyses of total Cu by ICP OES
235 detected no such effect in *N. gonorrhoeae* (13). As *N. gonorrhoeae* was killed by low nanomolar
236 doses of Cu(btsc) complexes (Tables 1 and 2), we reasoned that any gain in intracellular Cu
237 might remain below the detection limit of these measurements.
238 The analyses of Cu content were repeated here using *E. coli* because of its ability to
239 tolerate micromolar concentrations of Cu(gtsm) and Cu(atsm) (Table 2). First, we established
240 that treatment with 10 – 15 μM of ionic Cu salts increased the total Cu content of *E. coli* by 2 –
241 3-fold when compared with the untreated control (Figure 4A). These amounts of intracellular Cu
242 were non-inhibitory and there was no decrease in plating efficiency (Figure 4B). Treatment with
243 similar doses of Cu(atsm) induced a comparable rise in Cu levels (Figure 4A), again without any
244 loss in plating efficiency (Figures 3B). By contrast, exposure to equal doses of Cu(gtsm) led to a
245 greater accumulation of Cu that was 5 – 8-fold higher relative to the unchallenged control
246 (Figure 4A). This modest boost in intracellular Cu levels was correlated with an antibacterial
247 effect and there was a 5 – 10-fold reduction in plating efficiency (Figure 4B). There was no

change in the levels of other transition metal ions (Figure S3), confirming that the antibacterial effect of Cu(gtsm) was Cu-dependent.

Dissociation of Cu from Cu(btsc) complexes as bioavailable ions.

The relative ability of Cu(gtsm) and Cu(atsm) to promote intracellular accumulation of Cu matched their relative antibacterial potency (Table 2). In fact, for all bacterial pathogens that we tested, the MICs of Cu(gtsm) were invariably lower than those of Cu(atsm) (Table 2). This result was in line with the proposed action of Cu(btsc) complexes as Cu carriers. The Cu(II) center in Cu(btsc) is bound strongly and is not thought to be dissociated as Cu(II) ions. Instead, Cu is released as Cu(I) (see Figure 8 below). This occurs upon reduction of the Cu(II) center by biological reductants such as thiols (21). As a consequence of a higher Cu(II)/Cu(I) reduction mid-point potential for Cu(gtsm) (Figure 1), dissociation of Cu(I) ions from Cu(gtsm) is assured (21). By contrast, Cu(atsm) possesses a lower Cu(II)/Cu(I) mid-point potential, and thus dissociation of Cu(I) ions from Cu(atsm) is not thought to occur except in hypoxic cells (22, 23).

The final amounts of intracellular Cu delivered by Cu(gtsm) and Cu(atsm) (< 25 ng Cu/mg protein) were well below the maximum tolerable capacity of *E. coli*. Exposure to higher doses of Cu salts (1500 μM) led to the accumulation of Cu to 200 ng Cu/mg protein (Figure S3), but the plating efficiency of bacteria remained unchanged (Figure S1). This apparent disconnect between total Cu content and antibacterial potency has been observed previously (13, 15). Here it must be noted that ICP OES measurements do not differentiate between Cu that is captured by the bacterium as bioavailable ions and Cu that remains coordinated as a Cu(btsc) complex.

To examine the dissociation of Cu from Cu(btsc) complexes as bioavailable ions, we exploited the innate bacterial response to excess Cu. The system in *E. coli* is particularly well

271 characterized (Figure 5A) and amenable for analysis. In *E. coli*, increased intracellular Cu is
272 sensed by the Cu(I)-specific transcriptional regulator CueR. In turn, CueR activates the
273 expression of CopA, a membrane-bound P-type ATPase that exports Cu(I) out of the cytoplasm,
274 and CueO, a periplasmic cuprous oxidase that oxidizes Cu(I) to the less toxic form Cu(II) (Figure
275 5A) (24). In this work, we fused a plasmid-borne, promoterless *lacZ* transcriptional reporter gene
276 with the promoter region of the *copA* gene (P*copA-lacZ*, Figure 5A) and subsequently tested the
277 ability of ionic Cu salts, Cu(gtsm) and Cu(atsm) to induce β-galactosidase activity in *E. coli*.
278 This opportunity was not available with *N. gonorrhoeae*, as the CueR regulon or any other
279 recognizable Cu detoxification system is absent, with the sole exception of the efflux pump
280 CopA (25). Furthermore, unlike the *copA* gene in *E. coli*, expression of gonococcal *copA* is
281 controlled by an unidentified mechanism that does not appear to involve Cu (25).

282 Addition of Cu salts into the growth medium led to a dose-dependent increase in
283 β-galactosidase activity (Figure 5B). No induction was observed in the presence of other
284 transition metal ions (Figure 5C), thus validating the Cu-specific response of the P*copA-lacZ*
285 fusion. More importantly, exposure to Cu(gtsm) also led to a robust induction of β-galactosidase
286 activity (Figure 5B), consistent with the intracellular release of bioavailable Cu ions from
287 Cu(gtsm), presumably as Cu(I). While the minimum dose of Cu salts required for induction was
288 > 1 μM, activation by Cu(gtsm) was observed at concentrations as low as 0.1 μM (Figure 5B).
289 Moreover, activation of P*copA-lacZ* by Cu(gtsm) was rapid and the maximum response was
290 achieved as early as 20 min post-exposure (Figure 5D). There was a detectable decrease in this
291 response after 40 min, presumably due to the toxic effects of Cu(gtsm) or the dissociated Cu(I)
292 ion. By comparison, induction of P*copA-lacZ* by ionic Cu salts occurred gradually over a period

293 of at least 2 h (Figure 5D). These findings further confirmed that Cu(gtsm) is a more efficient
294 source of intracellular bioavailable Cu(I) ions than uncomplexed Cu salts.

295 As mentioned earlier, the Cu center in Cu(atsm) is considered to display less dissociation
296 intracellularly when compared to Cu(gtsm) (21-23). However, like Cu(gtsm), Cu(atsm) also
297 activated the P*copA* promoter as detected by an increase in β-galactosidase activity (Figure 5B).
298 The maximum magnitudes of activation by Cu(atsm) and Cu(gtsm) were comparable (Figures
299 4B and 4D). These results provided strong evidence that Cu was also released from Cu(atsm) as
300 bioavailable Cu(I) ions. However, when compared with Cu(gtsm), there was a reproducible lag
301 in the response to Cu(atsm) and a maximum was achieved only after 80 min of exposure (Figure
302 5D). Taken together, this data is consistent with the view that Cu(gtsm) is a more efficient Cu
303 delivery agent than Cu(atsm).
304
305 **Susceptibility of Cu tolerance mutants of *E. coli* to Cu(btsc) complexes and dissociation of**
306 **Cu ions in the cytoplasm.**

307 Activation of the P*copA* promoter by Cu(gtsm) and Cu(atsm) implied that expression of
308 *copA* and export of Cu(I) from the cytoplasm by CopA may mediate bacterial resistance to these
309 Cu complexes. To test this proposal, we examined whether inactivation of *copA* in *E. coli*
310 enhanced susceptibility to Cu(btsc) complexes. The effect of *copA* (and *cueO*) mutation on Cu
311 tolerance in *E. coli* has been well characterised previously (26, 27). In this work, we confirmed
312 that the *copA* mutant strain of *E. coli* was more sensitive to inhibition by Cu salts when
313 compared with the isogenic parent strain (Figure 6A), consistent with the established role of
314 CopA in tolerance to Cu ions. Importantly, the *copA* mutant was also sensitized to Cu(gtsm) and
315 no bacterial growth was observed above 5 μM (Figure 6B). This reduction in MIC when

316 compared with the wild type (MIC >25 μM, Table 2) supports a role for Cu(I) ion efflux by
317 CopA in the detoxification of Cu(gtsm).

318 A *cueO* mutant strain of *E. coli* that lacks the periplasmic cuprous oxidase (Figure 5A)
319 also displayed a Cu salt-sensitive phenotype (Figure 6A). However, susceptibility of the *cueO*
320 mutant to Cu(gtsm) was indistinguishable from that of the wild type (Figure 6B). This result
321 indicated that, although oxidation of toxic Cu(I) to Cu(II) by CueO was required for tolerance to
322 free Cu ions and salts, it was not essential for resistance to Cu(gtsm). While CopA protects
323 against cytoplasmic Cu toxicity, CueO operates in the periplasm (Figure 5A). Thus, the apparent
324 requirement for CopA but not CueO indicated that dissociation of bioavailable Cu ions from
325 Cu(gtsm) occurred specifically in the cytoplasm and not in the periplasm.

326 In the case of Cu(atsm), there was no reduction in its MIC against the *copA* mutant
327 (Figure 6C). However, there was a noticeable decrease in colony size (Figure 6C), which was
328 consistent with a suppressed growth rate in liquid medium (Figure S4). These observations
329 suggest that the CopA efflux pump may also confer tolerance to Cu(atsm), although the effect
330 was subtle presumably because generation of bioavailable Cu ions from this complex was
331 inefficient. As expected, Cu(atsm) had no observable effect on the *cueO* mutant (Figure 6C).

332 The toxic effects of salts of Cu and other metal ions are known to be affected severely by
333 speciation or potential binding and buffering by components of the culture medium (28). Thus, it
334 is often the case that the less complex the medium, the lower the MICs. The inhibitory effects of
335 Cu(gtsm) and Cu(atsm) on the growth of the most sensitive mutant, *copA*, were similar when
336 tested in LB or in M9 medium (Figure S4), suggesting that there was minimal release of free Cu
337 ions from Cu(btsc) complexes in the extracellular medium.

338

Efficacy of Cu(btsc) complexes against MDR strains of *N. gonorrhoeae*.

The demonstrated action of Cu(gtsm) and Cu(atsm) as carriers of Cu ions and the established mechanism of Cu ion poisoning by mis-metallation of enzymes and promotion of redox stress are distinct from the known modes of action of conventional antibiotics. Thus, we propose that these complexes may represent a promising new strategy for the treatment of antibiotic-resistant bacterial infections. Their low relative MICs against *N. gonorrhoeae* (Table 2) indicated that Cu(gtsm) and Cu(atsm) were highly potent against this bacterium. Therefore, we extended this work to test the potential for Cu(btsc) complexes to be used against multidrug-resistant (MDR) *N. gonorrhoeae*.

Cu(gtsm) showed robust activity against several antibiotic-resistant isolates of *N. gonorrhoeae* (Table 3), including the MDR strains F89 (29) and H041 (30), which are resistant to β-lactams (except carbapenems), fluoroquinolones, macrolides, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, chloramphenicol, and nitrofurantoin. The MICs for these isolates were comparable to that for the drug-susceptible strain 1291 (Table 3), suggesting that the antibacterial activity of Cu(gtsm) was not diminished by enzymes and transporters that confer resistance to other antibiotics. In *N. gonorrhoeae*, these include the pilus secretin PilQ (31) and the MtrFCDE multidrug efflux pump system (32). To test this hypothesis, we determined the MICs of Cu(gtsm) for strains KH15 and DW120, which are isogenic mutants of the drug-susceptible strain FA19 that express higher basal levels of the MtrCDE pump (32). Although these strains showed increased resistance to multiple antibiotics and antimicrobial peptides when compared with the parent strain (32), they were no less susceptible to Cu(gtsm) (Table 3).

Like Cu(gtsm), Cu(atsm) was also effective against MDR isolates and MtrFCDE-overexpressing strains of *N. gonorrhoeae* (Table 3). However, there were > 2-fold increases in MICs when compared to the antibiotic-susceptible strains (Table 3). This loss of efficacy indicated that Cu(atsm) may be a substrate for the MtrCDE efflux pump. Consistent with this proposal, inactivation of the *mtrD* gene (strain KH14) (33) led to a modest but reproducible decrease in the MIC of Cu(atsm) when compared to the isogenic parent strain (FA19) (Table 3). The loss of *mtrD* did not have any effect on the MIC of Cu(gtsm) (Table 3).

Viability of host epithelial cells in the presence of antimicrobial doses of Cu(btsc) complexes.

To ascertain the potential of Cu and Cu(btsc) complexes as a clinically useful anti-gonococcal agent, we examined whether Cu(gtsm) and Cu(atsm) exert an effect on the viability of cervical epithelial cells (ME-180 epithelial cells) *in vitro*. As shown in Figure 7, incubation of ME-180 monolayers with up to 0.5 μM of Cu(gtsm) and 5 μM of Cu(atsm) (ca. 5X the MIC against *N. gonorrhoeae*, see Table 3) for 24 h did not result in loss of cell viability. There was no loss in cell numbers (Figure 7) and more than 95% of these cells retained the ability to exclude Trypan Blue (Figure 7). Under our experimental conditions, the ME-180 cell line did not withstand treatment with DMSO beyond 0.1 v/v %. Thus, higher concentrations of the Cu complexes were not tested because of poor solubility in the culture media without DMSO. Nevertheless, previous work with human prostate epithelial cells were had used up to 100 μM without any observable loss of viability (34), indicating a potential therapeutic index of 1000 for *N. gonorrhoeae* These results indicate that at antimicrobial doses, the test compounds exert minimal toxicity towards host cells.

DISCUSSION

Insight into the mode of antibacterial action of Cu(btsc) complexes.

Dynamics of "Cu-boosting" by Cu(btsc) complexes. The use of lipophilic ligands to deliver metal ions into cells is an established concept, particularly in the detection and intervention of cancers (35). In terms of antimicrobial applications, this concept has been demonstrated by the use of zinc pyrithione (Zn-PYT) as an antifungal in soaps and shampoos. The mode of action of Zn-PYT depends on transchelation with free Cu ions, presumably from the extracellular environment, and subsequent delivery of these Cu ions into the target organism (36). Coordination complexes that act as carriers for Cu ions are now being increasingly investigated as a novel approach to combat bacterial infections (9, 13-15).

The bis(thiosemicarbazone) family of Cu carriers displays anticancer activities (37). There is also interest in their potential as neurotherapeutics and as imaging agents for hypoxia. Our group and others have now demonstrated that Cu(btsc) complexes are also promising antimicrobials (13-15). However, while studies with mammalian systems have established the action of these complexes as agents that alter the bioavailability of Cu, studies with bacteria have not been equally conclusive, as the final Cu content of treated bacteria did not always correlate with survival or viability (13, 15). These studies have focused on the total amounts of Cu at the 'end point' (*eg.* the ICP OES measurements in Figure 4A) but the present work suggested that the kinetics of Cu influx and potential efflux by detoxification systems must also be considered (Figure 8). This model is likely to be universal to all lipophilic Cu carriers and not limited to those containing btsc ligands.

Our results using *E. coli* showed that both Cu(gtsm) and Cu(atsm) enter the bacterial cytoplasm more rapidly than do ionic Cu salts. These complexes are membrane-permeable, probably *via* passive diffusion as an uptake system has not been identified (38). As these complexes are uncharged, they would equilibrate rapidly across bacterial membranes and would not accumulate as intact molecules to a high intracellular concentration. However, reduction of the Cu(II) center and subsequent dissociation as bioavailable Cu(I) ion would generate a powerful mass-action effect (Figure 8). This thermodynamically-driven influx of Cu ions may overwhelm basal Cu tolerance and cause Cu poisoning. By comparison, the more restricted entry of ionic Cu salts may allow activation of dedicated Cu detoxification mechanisms, which would enable the bacterial cell to amass and, more importantly, survive higher final amounts of total Cu.

The above model extends to the observed difference between the antibacterial activities of Cu(gtsm) and Cu(atsm). While studies with mammalian systems suggest little intracellular dissociation of bioavailable Cu ions from Cu(atsm) (21-23), our work with *E. coli* indicated that it does occur, although it is less efficient than the equivalent process from Cu(gtsm). We cannot discount possible variations in the rates of membrane penetration by the two complexes as a result of subtle differences in lipophilicity (39). Nevertheless, it is more likely that, as a consequence of a lower Cu(II)/Cu(I) mid-point potential for Cu(atsm) (Figure 1), the rate of reduction (and, subsequently, dissociation) of Cu from Cu(atsm) is also lower. The bacterial cell thus has more time to respond and detoxify the excess Cu, resulting in a lower antibacterial potency of Cu(atsm).

Our results with MtrCDE-overexpressing strains of *N. gonorrhoeae* (Table 3) also indicate that potential efflux of Cu(gtsm) and Cu(atsm) as intact molecules out of the cytoplasm

must not be overlooked. Cu(gtsm) rapidly and efficiently dissociates within the cytoplasm and thus this complex may evade active export by promiscuous efflux transporters. By comparison, Cu(atsm) may linger as an intact molecule and thus be exported prior to dissociation and subsequent release of bioavailable Cu(I) ions (Figure 8). This removal of Cu(atsm) from the cytoplasm, either by MtrCDE or other efflux systems, would further reduce its antibacterial efficacy. Although we have not tested this idea directly, the AcrAB-TolC multi-drug efflux pump system (ref) it is likely that in this transporter will contribute to the observed resistance of this bacterium to Cu(atsm) (40).

Correlation between bacterial physiology and susceptibility to Cu(btsc) complexes. The antibacterial activity of Cu(btsc) complexes has now been tested against several important human pathogens, including *S. pneumoniae*, *H. influenzae*, uropathogenic *E. coli*, *Salmonella* (Table 2), as well as *M. tuberculosis* (14) and *S. aureus* (15). Not all of these showed equal promise as targets for Cu(btsc) therapeutics, but all showed less susceptibility when compared with *N. gonorrhoeae.* The explanation for these differences may relate to bacterial physiology.

Compared with most other bacterial pathogens, *N. gonorrhoeae* possesses a Cu detoxification system that is unusually underdeveloped. It consists of a single Cu efflux pump, CopA, and no additional cytoplasmic or periplasmic accessories (25). Importantly, Cu does not induce the expression of the *copA* gene. Thus, while gonococcal CopA may participate in general maintenance of Cu levels during regular metabolism (25, 41), it may be unable to confer resistance to severe Cu stress. This absence of an inducible resistance system coincides with the availability of targets of poisoning by Cu or Cu(btsc) complexes (Figure 8). These targets include iron-sulfur (Fe-S) cluster-containing enzymes such as coproporhyrinogen(III) oxidase in the pathway for heme biosynthesis (41). In addition, *N. gonorrhoeae* depends on two NADH

453 dehydrogenases that are both susceptible to inhibition by Cu(btsc) complexes (13). As a
454 consequence, *N. gonorrhoeae* displays hypersensitivity to inhibition by Cu salts and Cu(btsc)
455 complexes, particularly Cu(gtsm) (Tables 1 – 3).

456 In *E. coli*, major targets of Cu poisoning are available, such as the Fe-S cluster enzymes
457 fumarase in the TCA cycle and isopropylmalate dehydratase in the pathway for branched-chain
458 amino acid synthesis (42). However, this bacterium also possesses sophisticated and robust,
459 inducible defenses against Cu toxicity (24). In addition, *E. coli* uses a versatile respiratory
460 electron transport system and fermentative systems that are less sensitive to Cu(btsc) complexes.
461 These arguments correlate well with our finding that *E. coli* is more resistant to inhibition by
462 Cu(btsc) complexes, even Cu(gtsm) (Table 2). Similarly, the Cu detoxification system in *S.*
463 *pneumoniae* is relatively well-developed compared with the system in *N. gonorrhoeae*. However,
464 *S. pneumoniae* has a relatively low dependence on Fe-S cluster enzymes and it does not contain a
465 respiratory chain. This may explain the limited sensitivity of this bacterium to Cu(atsm) and
466 Cu(gtsm) (Table 2). For these Cu-tolerant bacteria, the antimicrobial efficacy of Cu delivery
467 agents might be enhanced if used in conjunction with a CopA antagonist to trap excess Cu ions.
468
469 **Cu delivery agents as a novel concept for the topical treatment of *N. gonorrhoeae.***

470 Gonorrhea is the second most prevalent sexually transmissible infection worldwide and
471 management of this disease represents significant challenge to public health. There is no vaccine
472 and thus antibiotic treatment remains the only method to control the spread of infection.
473 However, MDR strains have developed resistance to virtually all first-line antibiotics (43). Our
474 work suggests that delivery of bioavailable Cu ions may represent a new approach to combat
475 gonococcal infections. The application of copper in the cervix and vagina is an established

476 concept and intrauterine devices containing elemental copper is one of the most common and
477 most effective non-hormonal contraceptives worldwide (44). Here we showed that Cu salts and
478 Cu(btsc) complexes did not affect the viability of cervical epithelial cells *in vitro* at
479 concentrations that were inhibitory to the gonococcus (Figure 7). Moreover, Cu salts and
480 Cu(btsc) complexes were ineffective against lactic acid bacteria, as exemplified by *Lactobacillus*
481 *acidophilus* (see Table 2), suggesting that Cu delivery agents can be used to target gonococci
482 without significantly affecting the commensal flora. Crucially, unlike other bacterial pathogens
483 that have been identified as potential targets for treatments by Cu delivery agents, including *M.*
484 *tuberculosis* (14), *S. aureus* (15), and *Cryptococcus neoformans* (9), *N. gonorrhoeae* is primarily
485 an extracellular mucosal pathogen that colonises surfaces of the genitourinary epithelium.
486 Gonococcal infections are thus amenable to topical drug formulations and would bypass many of
487 the challenges of a systemic route for the delivery of Cu.
488
489 **ACKNOWLEDGEMENT**
490 We acknowledge National Health and Medical Research Council (Australia) Program
491 Grant 565526 to AGM and NIH grant R21 AI 103270-01A1 and a VA Merit Award
492 (Department of Veterans Affairs Medical Research Service) to WMS. KYD is supported by an
493 Endeavour Research Fellowship (Department of Education and Training, Australia). WMS is the
494 recipient of a Senior Research Career Scientist Award from the VA Medical Research Service.
495 MAS is supported by an Australian Research Council Future Fellowship (FT100100662). The
496 contents of this article is solely the responsibility of the authors and does not necessarily rflect
497 the official views of the National Institutes of Health or the Department of Veterans Affairs.

We thank B. Paterson (The University of Melbourne, Australia) for synthesis of Cu(btsc) compounds, and M. Mostert (The University of Queensland, Australia) for technical assistance with ICP OES measurements. We are also grateful to M. Achard (The University of Queensland, Australia) for construction of *E. coli* mutant strains, M. Turner (The University of Queensland, Australia) for providing *L. acidophilus*, R. Nicholas (University of North Carolina-Chapel Hill) for *N. gonorrhoeae* strains F89 and HO41, and S. Satola (Emory University) for *S. aureus* strains.

CORRESPONDENCE

Address all correspondence to KYD (k.djoko@uq.edu.au) or AGM (mcewan@uq.edu.au). The authors declare no competing financial interest.

REFERENCES

1. **Xiao Z, Loughlin F, George GN, Howlett GJ, Wedd AG.** 2004. C-terminal domain of the membrane copper transporter Ctr1 from Saccharomyces cerevisiae binds four Cu(I) ions as a cuprous-thiolate polynuclear cluster: sub-femtomolar Cu(I) affinity of three proteins involved in copper trafficking. J Am Chem Soc **126:**3081-3090.

2. **Foster AW, Osman D, Robinson NJ.** 2014. Metal preferences and metallation. J Biol Chem **289:**28095-28103.

3. **Valko M, Morris H, Cronin MTD.** 2005. Metals, toxicity and oxidative stress. Curr Med Chem **12:**1161-1208.

4. **Osman D, Cavet JS.** 2008. Copper homeostasis in bacteria. Adv Appl Microbiol **65:**217-247.

5. **Dollwet HHA, Sorenson JRJ.** 1985. Historic uses of copper-compounds in medicine. Trace Elements in Medicine **2:**80-87.

6. **Galhardi CM, Diniz YS, Faine LA, Rodrigues HG, Burneiko RC, Ribas BO, Novelli EL.** 2004. Toxicity of copper intake: lipid profile, oxidative stress and susceptibility to renal dysfunction. Food Chem Toxicol **42:**2053-2060.

7. **Santo CE, Lam EW, Elowsky CG, Quaranta D, Domaille DW, Chang CJ, Grass G.** 2011. Bacterial killing by dry metallic copper surfaces. Applied and Environmental Microbiology **77:**794-802.

8. **Grass G, Rensing C, Solioz M.** 2011. Metallic copper as an antimicrobial surface. Applied and Environmental Microbiology **77:**1541-1547.

9. **Festa RA, Helsel ME, Franz KJ, Thiele DJ.** 2014. Exploiting innate immune cell activation of a copper-dependent antimicrobial agent during infection. Chem Biol **21:**977-987.

10. **White C, Lee J, Kambe T, Fritsche K, Petris MJ.** 2009. A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. J Biol Chem **284:**33949-33956.

11. **Subashchandrabose S, Hazen TH, Brumbaugh AR, Himpsl SD, Smith SN, Ernst RD, Rasko DA, Mobley HLT.** 2014. Host-specific induction of Escherichia coli fitness genes during human urinary tract infection. Proc Natl Acad Sci U S A **111:**18327-18332.

12. **Helsel ME, Franz KJ.** 2015. Pharmacological activity of metal binding agents that alter copper bioavailability. Dalton Trans doi:10.1039/c5dt00634a.

13. **Djoko KY, Paterson BM, Donnelly PS, McEwan AG.** 2014. Antimicrobial effects of copper(II) bis(thiosemicarbazonato) complexes provide new insight into their biochemical mode of action. Metallomics **6:**854-863.

14. **Speer A, Shrestha TB, Bossmann SH, Basaraba RJ, Harber GJ, Michalek SM, Niederweis M, Kutsch O, Wolschendorf F.** 2013. Copper-boosting compounds: a novel concept for antimycobacterial drug discovery. Antimicrob Agents Chemother **57:**1089-1091.

15. **Haeili M, Moore C, Davis CJ, Cochran JB, Shah S, Shrestha TB, Zhang Y, Bossmann SH, Benjamin WH, Kutsch O, Wolschendorf F.** 2014. Copper complexation screen reveals compounds with potent antibiotic properties against methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother **58:**3727-3736.

16. **Xiao Z, Brose J, Schimo S, Ackland SM, La Fontaine S, Wedd AG.** 2011. Unification of the copper(I) binding affinities of the metallo-chaperones Atx1, Atox1, and related proteins: detection probes and affinity standards. J Biol Chem **286:**11047-11055.

17. **Farinha MA, Kropinski AM.** 1990. Construction of broad-host-range plasmid vectors for easy visible selection and analysis of promoters. J Bacteriol **172:**3496-3499.

18. **Djoko KY, Donnelly PS, McEwan AG.** 2014. Inhibition of respiratory Complex I by copper(II)-bis(thiosemicarbazonato) complexes. Metallomics **6:**2250-2259.

19. **Calhoun MW, Gennis RB.** 1993. Demonstration of separate genetic loci encoding distinct membrane-bound respiratory NADH dehydrogenases in *Escherichia coli*. J Bacteriol **175:**3013-3019.

20. **Ueno H, Miyoshi H, Inoue M, Niidome Y, Iwamura H.** 1996. Structural factors of rotenone required for inhibition of various NADH-ubiquinone oxidoreductases. Biochim Biophys Acta **1276:**195-202.

21. **Xiao ZG, Donnelly PS, Zimmermann M, Wedd AG.** 2008. Transfer of copper between bis(thiosemicarbazone) ligands and intracellular copper-binding proteins. Insights into mechanisms of copper uptake and hypoxia selectivity. Inorg Chem **47:**4338-4347.

22. **Donnelly PS, Caragounis A, Du T, Laughton KM, Volitakis I, Cherny RA, Sharples RA, Hill AF, Li QX, Masters CL, Barnham KJ, White AR.** 2008. Selective intracellular release of copper and zinc ions from bis(thiosemicarbazonato) complexes reduces levels of Alzheimer disease amyloid-beta peptide. J Biol Chem **283:**4568-4577.

23. **Obata A, Yoshimi E, Waki A, Lewis JS, Oyama N, Welch MJ, Saji H, Yonekura Y, Fujibayashi Y.** 2001. Retention mechanism of hypoxia selective nuclear imaging/radiotherapeutic agent cu-diacetyl-bis(N4-methylthiosemicarbazone) (Cu-ATSM) in tumor cells. Ann Nucl Med **15:**499-504.

24. **Rensing C, Grass G.** 2003. *Escherichia coli* mechanisms of copper homeostasis in a changing environment. FEMS Microbiol Rev **27:**197-213.

25. **Djoko KY, Franiek JA, Edwards JL, Falsetta ML, Kidd SP, Potter AJ, Chen NH, Apicella MA, Jennings MP, McEwan AG.** 2012. Phenotypic characterization of a *copA* mutant of *Neisseria gonorrhoeae* identifies a link between copper and nitrosative stress. Infect Immun **80:**1065-1071.

26. **Grass G, Rensing C.** 2001. Genes involved in copper homeostasis in *Escherichia coli*. J Bacteriol **183:**2145-2147.

27. **Rensing C, Fan B, Sharma R, Mitra B, Rosen BP.** 2000. CopA: An *Escherichia coli* Cu(I)-translocating P-type ATPase. Proc Natl Acad Sci U S A **97:**652-656.

585 28. **Haase H, Hebel S, Engelhardt G, Rink L.** 2015. The biochemical effects of
586 extracellular Zn(2+) and other metal ions are severely affected by their speciation in cell culture
587 media. Metallomics **7:**102-111.
588 29. **Unemo M, Golparian D, Nicholas R, Ohnishi M, Gallay A, Sednaoui P.** 2012. High-
589 level cefixime- and ceftriaxone-resistant *Neisseria gonorrhoeae* in France: novel *penA* mosaic
590 allele in a successful international clone causes treatment failure. Antimicrob Agents Chemother
591 **56:**1273-1280.
592 30. **Shimuta K, Unemo M, Nakayama S, Morita-Ishihara T, Dorin M, Kawahata T,**
593 **Ohnishi M.** 2013. Antimicrobial resistance and molecular typing of *Neisseria gonorrhoeae*
594 isolates in Kyoto and Osaka, Japan, 2010 to 2012: intensified surveillance after identification of
595 the first strain (H041) with high-level ceftriaxone resistance. Antimicrob Agents Chemother
596 **57:**5225-5232.
597 31. **Whiley DM, Jacobsson S, Tapsall JW, Nissen MD, Sloots TP, Unemo M.** 2010.
598 Alterations of the *pilQ* gene in *Neisseria gonorrhoeae* are unlikely contributors to decreased
599 susceptibility to ceftriaxone and cefixime in clinical gonococcal strains. J Antimicrob Chemother
600 **65:**2543-2547.
601 32. **Hagman KE, Pan W, Spratt BG, Balthazar JT, Judd RC, Shafer WM.** 1995.
602 Resistance of *Neisseria gonorrhoeae* to antimicrobial hydrophobic agents is modulated by the
603 *mtrRCDE* efflux system. Microbiology **141 (Pt 3):**611-622.
604 33. **Hagman KE, Lucas CE, Balthazar JT, Snyder L, Nilles M, Judd RC, Shafer WM.**
605 1997. The MtrD protein of *Neisseria gonorrhoeae* is a member of the
606 resistance/nodulation/division protein family constituting part of an efflux system. Microbiology
607 **143 (Pt 7):**2117-2125.

34. **Cater MA, Pearson HB, Wolyniec K, Klaver P, Bilandzic M, Paterson BM, Bush AI, Humbert PO, La Fontaine S, Donnelly PS, Haupt Y.** 2013. Increasing Intracellular Bioavailable Copper Selectively Targets Prostate Cancer Cells. ACS Chem Biol doi:10.1021/cb400198p.

35. **Tisato F, Marzano C, Porchia M, Pellei M, Santini C.** 2010. Copper in diseases and treatments, and copper-based anticancer strategies. Med Res Rev **30:**708-749.

36. **Reeder NL, Kaplan J, Xu J, Youngquist RS, Wallace J, Hu P, Juhlin KD, Schwartz JR, Grant RA, Fieno A, Nemeth S, Reichling T, Tiesman JP, Mills T, Steinke M, Wang SL, Saunders CW.** 2011. Zinc pyrithione inhibits yeast growth through copper influx and inactivation of iron-sulfur proteins. Antimicrob Agents Chemother **55:**5753-5760.

37. **Paterson BM, Donnelly PS.** 2011. Copper complexes of bis(thiosemicarbazones): from chemotherapeutics to diagnostic and therapeutic radiopharmaceuticals. Chem Soc Rev **40:**3005-3018.

38. **Price KA, Crouch PJ, Volitakis I, Paterson BM, Lim S, Donnelly PS, White AR.** 2011. Mechanisms controlling the cellular accumulation of copper *bis*(thiosemicarbazonato) complexes. Inorg Chem **50:**9594-9605.

39. **Dearling JL, Lewis JS, Mullen GE, Welch MJ, Blower PJ.** 2002. Copper bis(thiosemicarbazone) complexes as hypoxia imaging agents: structure-activity relationships. J Biol Inorg Chem **7:**249-259.

40. **Okusu H, Ma D, Nikaido H.** 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of Escherichia coli multiple-antibiotic-resistance (Mar) mutants. J Bacteriol **178:**306-308.

630 41. **Djoko KY, McEwan AG.** 2013. Antimicrobial action of copper is amplified via
631 inhibition of heme biosynthesis. ACS Chem Biol doi:10.1021/cb4002443.
632 42. **Macomber L, Imlay JA.** 2009. The iron-sulfur clusters of dehydratases are primary
633 intracellular targets of copper toxicity. Proc Natl Acad Sci U S A **106:**8344-8349.
634 43. **Unemo M, Shafer WM.** 2014. Antimicrobial resistance in *Neisseria gonorrhoeae* in the
635 21st century: past, evolution, and future. Clin Microbiol Rev **27:**587-613.
636 44. **d'Arcangues C.** 2007. Worldwide use of intrauterine devices for contraception.
637 Contraception **75:**S2-7.
638 45. **Apicella MA.** 1974. Antigenically distinct populations of Neisseria gonorrhoeae:
639 isolation and characterization of the responsible determinants. J Infect Dis **130:**619-625.
640 46. **Nachamkin I, Cannon JG, Mittler RS.** 1981. Monoclonal antibodies against Neisseria
641 gonorrhoeae: production of antibodies directed against a strain-specific cell surface antigen.
642 Infect Immun **32:**641-648.
643 47. **Schneider H, Griffiss JM, Williams GD, Pier GB.** 1982. Immunological basis of serum
644 resistance of Neisseria gonorrhoeae. J Gen Microbiol **128:**13-22.
645 48. **Swanson J, Barrera O, Sola J, Boslego J.** 1988. Expression of outer membrane protein
646 II by gonococci in experimental gonorrhea. J Exp Med **168:**2121-2129.
647 49. **Danielsson D, Faruki H, Dyer D, Sparling PF.** 1986. Recombination near the antibiotic
648 resistance locus penB results in antigenic variation of gonococcal outer membrane protein I.
649 Infect Immun **52:**529-533.
650 50. **Maness MJ, Sparling PF.** 1973. Multiple antibiotic resistance due to a single mutation
651 in Neisseria gonorrhoeae. J Infect Dis **128:**321-330.
652
653

TABLES

Table 1. Antibacterial activity of several Cu complexes against *N. gonorrhoeae*.

Complex[a]	MIC (μM)
Cu(gtsm)	0.10
Cu-pyrithione	0.13
Cu(atsm)	0.80
Cu-neocuproine	0.80
Cu-disulfiram	15 – 20
Cu salt	250

[a]The complexes were prepared and their concentrations standardised as described in Materials and Methods.

662 **Table 2.** Susceptibility of select bacterial pathogens to Cu(atsm) and Cu(gtsm).

663

Organism[a]	MIC (μM)	
	Cu(atsm)	**Cu(gtsm)**
N. gonorrhoeae	0.8	0.1
H. influenzae	10	1
S. aureus	>10	1.5
S. pneumoniae	>10	2
L. acidophilus	>25	5
S. Typhimurium	>25	>25
E. coli	>25	>25

664

665 [a]Strain information is available in Materials and Methods.

666

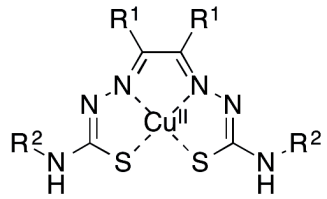
667 **Table 3.** Susceptibility of multidrug-resistant strains of *N. gonorrhoeae* to Cu salt, Cu(atsm) and
668 Cu(gtsm). Data presented were averaged from three independent experiments. Standard
669 deviations from the mean are shown in brackets.
670
671

	Strain[a]	Ref	MIC (μM)		
			Cu salt	Cu(atsm)	Cu(gtsm)
antibiotic-sensitive	1291	(45)	250 (0)	0.8 (0.1)	0.1 (0.0)
	FA1090	(46)	250 (0)	0.9 (0.1)	0.1 (0.0)
	F62	(47)	250 (0)	0.7 (0.1)	0.1 (0.0)
antibiotic-resistant	MS11	(48)	250 (0)	2.3 (0.2)	0.1 (0.0)
	FA6140	(49)	250 (0)	3.0 (0.3)	0.1 (0.2)
	F89	(29)	250 (0)	1.7 (0.3)	0.1 (0.0)
	H041	(30)	250 (0)	1.9 (0.2)	0.1 (0.0)
	FA19	(50)	250 (0)	0.9 (0.1)	0.1 (0.0)
	DW120	(32)	250 (0)	2.2 (0.2)	0.1 (0.0)
	KH15	(32)	250 (0)	2.7 (0.3)	0.1 (0.0)
	KH14	(33)	250 (0)	0.7 (0.1)	0.1 (0.0)

672
673 [a]Antibiotic resistance profiles of select strains are shown in Table S1.
674

675 **FIGURES**

676



Cu(gtsm): R^1= H; R^2 = CH_3
$E_{1/2}$ = -440 mV

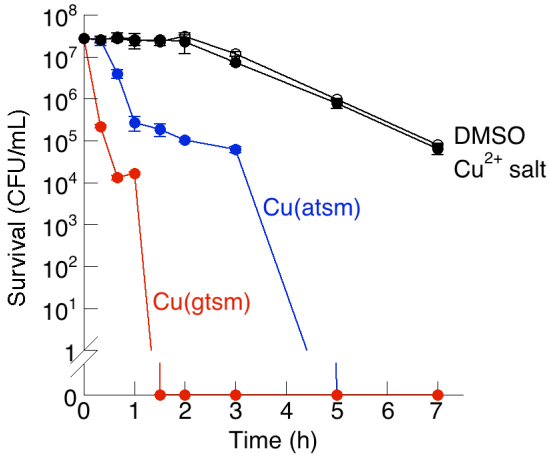
Cu(atsm): R^1= R^2 = CH_3
$E_{1/2}$ = -600 mV

677

678 **Figure 1.** Structure of Cu(btsc) complexes. Mid-point reduction potentials are *vs* Ag/AgCl.[21]

679

680

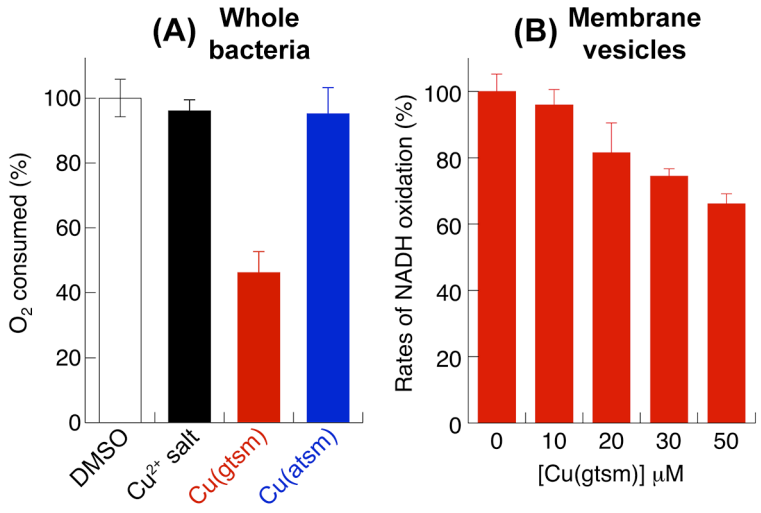


681

682

683 **Figure 2.** Time-dependent bactericidal effects of Cu(btsc) complexes (1 μM each) against *N.*
684 *gonorrhoeae* 1291. Number of surviving CFUs was plotted against time post-challenge. The
685 effects of Cu salt (1 μM) and DMSO control were also shown. Each data point was averaged
686 from three independent replicates. Error bars represent ± standard deviation from the mean.

687



688

689

690 **Figure 3.** Inhibitory effects of Cu(btsc) complexes on aerobic respiration by *E. coli.* **(A)**
691 Amounts of O_2 consumed by whole bacteria over 15 min in the presence of various Cu sources
692 (50 μM). **(B)** Rates of NADH oxidation by isolated membrane vesicles in the presence of
693 Cu(gtsm) (0 – 50 μM). **(A and B)** Results were shown as a percentage of the unchallenged
694 control. Each data point was averaged from three independent replicates. Error bars represent ±
695 standard deviation from the mean.

696

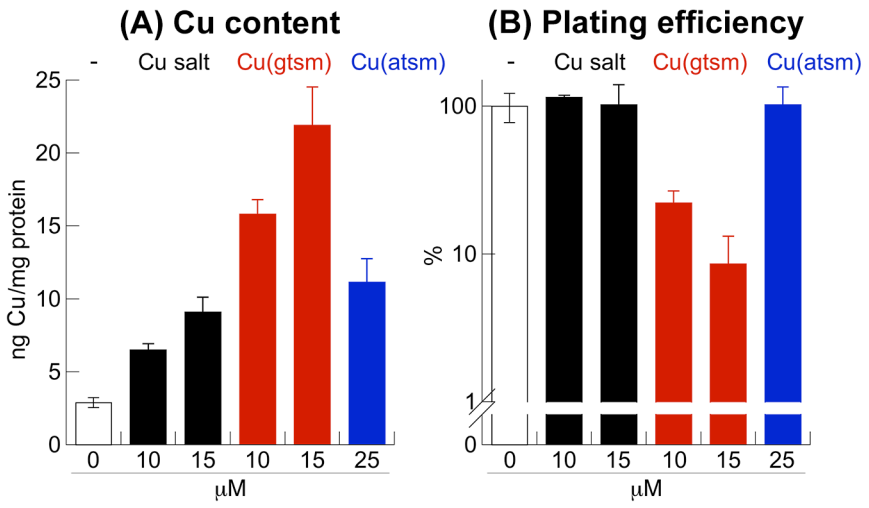
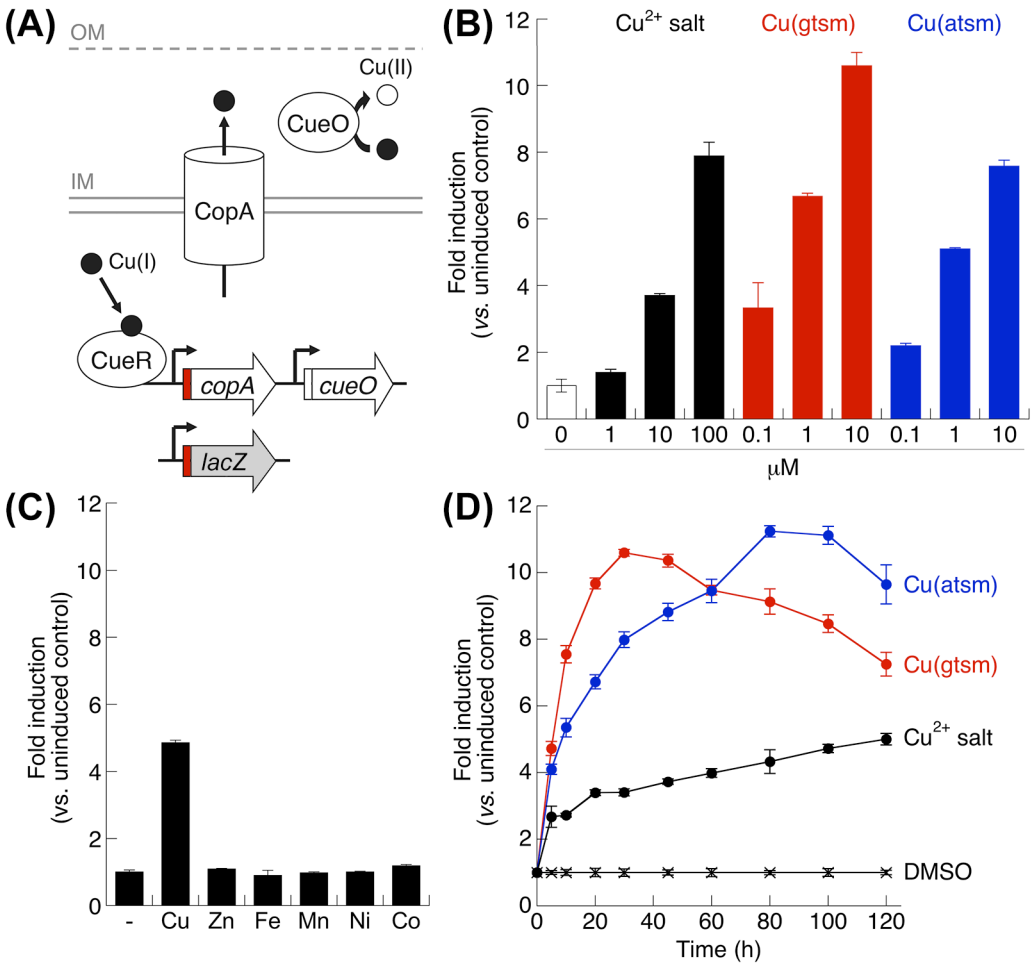


Figure 4. Effects of Cu(btsc) complexes on **(A)** total intracellular Cu content and **(B)** plating efficiency of *E. coli*, presented as a percentage of *vs.* the unchallenged control. **(A and B)** Treatment time was 24 h. Each data point was averaged from three independent replicates. Error bars represent ± standard deviation from the mean.



704

705

706 **Figure 5.** Release of Cu ions from Cu(btsc) complexes. **(A)** Cu detoxification system in *E. coli*.
707 To determine the presence of bioavailable Cu(I) ions in the cytoplasm, the promoter region of
708 *copA* (in red) was fused to a promoterless *lacZ* gene (in grey). IM, inner membrane; OM, outer
709 membrane. **(B)** Response of P*copA-lacZ* fusion to divalent transition metal ions (supplied as
710 chloride salt, 100 μM each) at t = 2 h post-exposure. **(C)** Dose-dependent induction of the *copA*
711 promoter. β-galactosidase activity was assayed at t = 2 h post-exposure. **(D)** Time-dependent
712 induction of the *copA* promoter in response to Cu salt (100 μM), Cu(gtsm) (10 μM) or Cu(atsm)
713 (25 μM). **(B – D)** Each data point was averaged from three replicates. Error bars represent ±

714 standard deviation from the mean. The results shown were representative of at least three
715 independent experiments.
716

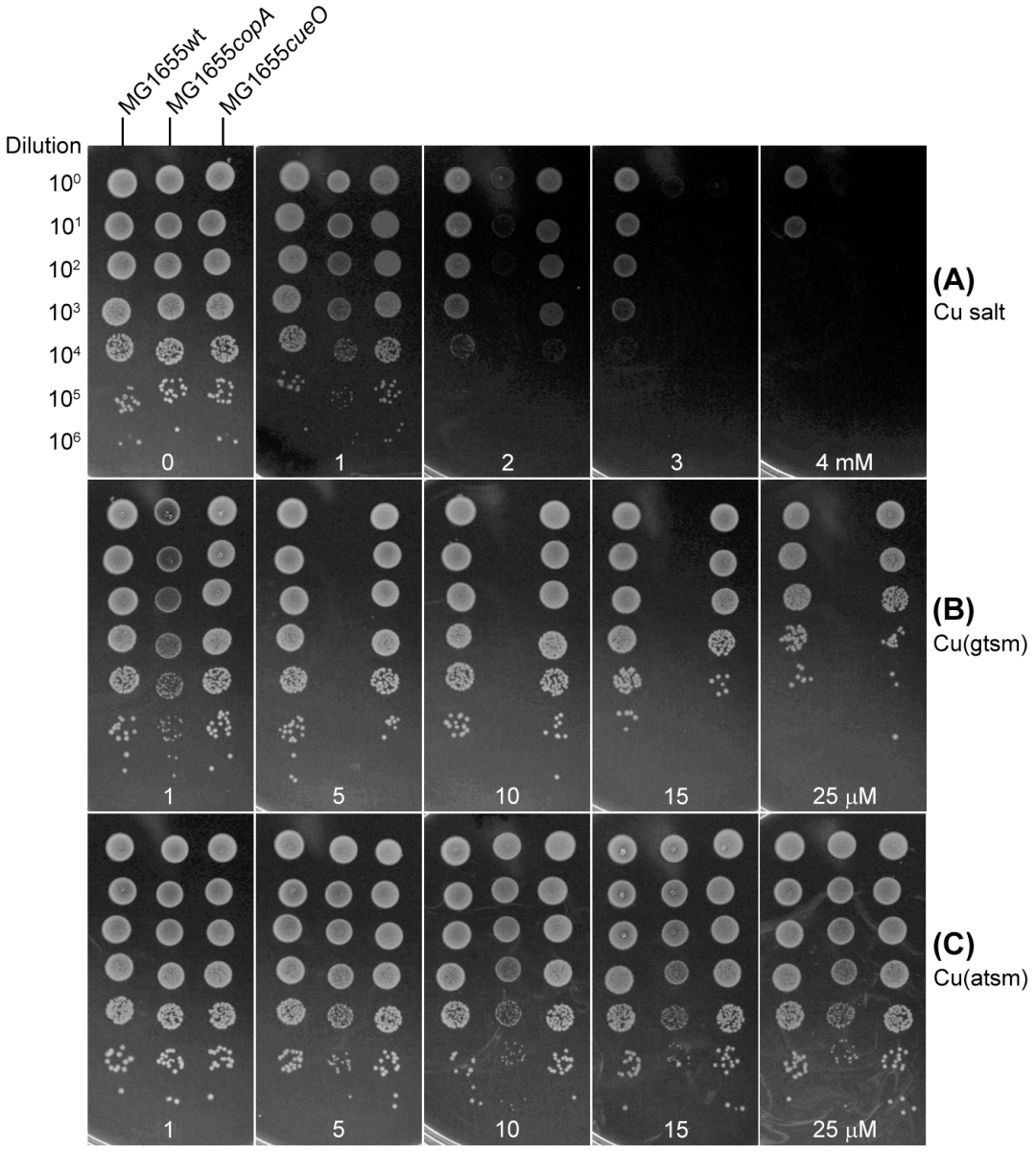
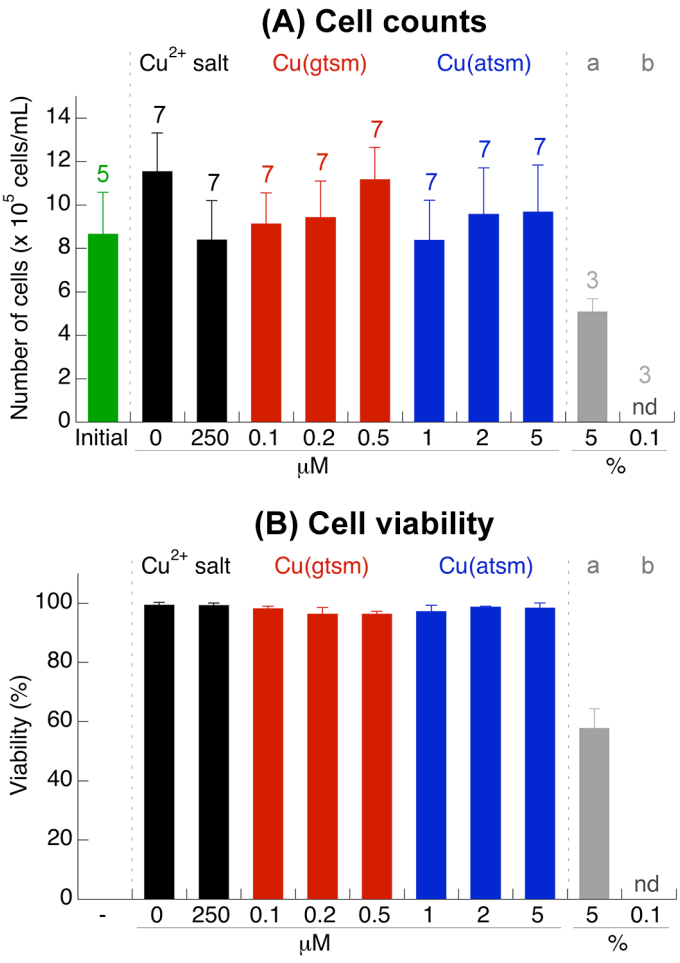


Figure 6. Susceptibility of *E. coli* mutant strains to **(A)** Cu salt, **(B)** Cu(gtsm), and **(C)** Cu(atsm) as determined by efficiency of plating on solid medium. Serial dilutions of bacteria were shown on the left. Concentrations of the various Cu sources were indicated at the bottom. Treatment time was 24 h. The results shown were representative of at least three independent experiments.

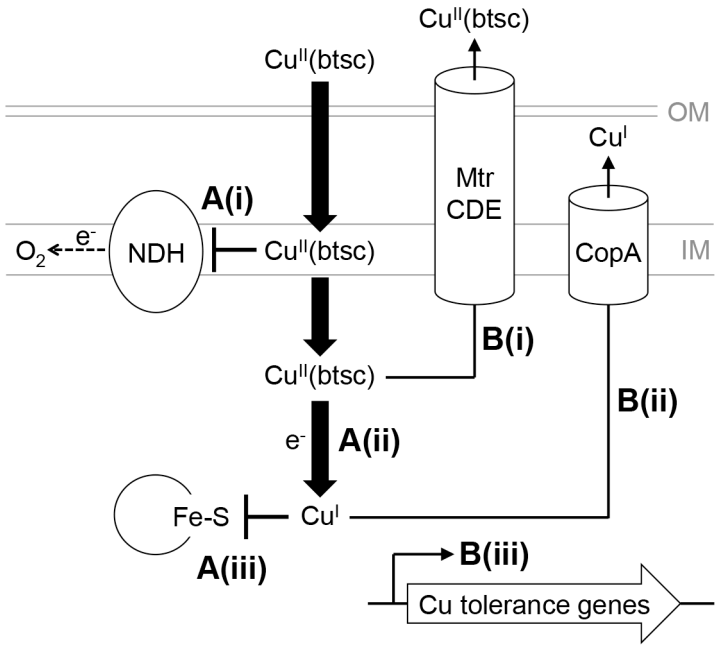


724

725

726 **Figure 7.** Effects of Cu(btsc) complexes on **(A)** total cell counts and **(B)** cell viability of ME-180
727 cervical epithelial cells. The treatment time was 24 h. The viability of cells in panel **B** was
728 determined using the same corresponding set of cells used for counting in panel **A**. The number
729 of replicates is shown above each column. Error bars represent ± standard deviation from the
730 mean. There was no statistically significant difference in the cell counts of viability from the
731 different treatments. The results shown were representative of at least three independent
732 experiments. DMSO (5 v/v %, columns a) and Triton X-100 (0.1 v/v %, columns b) were also
733 included as positive controls of cell death.

734



735

736

737 **Figure 8.** Schematic representation for the antibacterial action of Cu(btsc) complexes. **A.**
738 Mechanisms of action: **(i)** Inhibition of membrane-bound NADH dehydrogenases of the electron
739 transport chain by the intact Cu(btsc) complex, **(ii)** reduction of the Cu^{II} centre and dissociation
740 of Cu^{I} as bioavailable ions, and **(iii)** poisoning of enzymes by Cu^{I} ions. **B.** Mechanisms of
741 tolerance: **(i)** Efflux of the intact Cu(btsc) complex by the MtrCDE efflux pump or other
742 promiscuous drug transporters, **(ii)** efflux of bioavailable Cu^{I} ions, and **(iii)** activation of other
743 dedicated Cu ion tolerance genes.